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Light Dependent Endolysosomal Defects in a Photoreceptor Model of Alzheimer's Disease

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Light Dependent Endolysosomal Defects in a Photoreceptor Model of Alzheimer's Disease

A Thesis

Presented to

The Faculty and the Honors Program

Of the University of San Diego

By

Michelle Sydney Smith

Biology

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Honors Thesis Approval Page

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Title of Thesis: __Light Dependent Endolysosomal Defects in a Photoreceptor Model of
Alzheimer's Disease_

Accepted by the Honors Program and faculty of the Department of _____Biology_____,
University of San Diego, in partial fulfillment of the requirements for the Degree of Bachelor of
Arts. [or other degree]

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disease which is the 6th leading cause of death in the US. AD pathology is thought to be linked to the accumulation and aggregation of toxic proteins, amyloid-beta and tau. AD development and neurodegeneration is proposed to be caused by the toxic effects of these protein accumulations, specifically amyloid-beta, as postulated by the amyloid-cascade hypothesis. To study the relationship between amyloid-beta and overall neuronal health, a study was carried out using an amyloid-expressing fruit fly photoreceptor model. Using this model, toxicity of amyloid in a stressed lysosomal system induced by light, an established lysosomal stressor to the fly eye, was assessed. Exposure to light increases neurodegeneration in amyloid-expressing photoreceptors. Furthermore, light exposure normally induces endocytosis and lysosomal degradation of rhodopsin, but rhodopsin accumulates in intracellular puncta in amyloid-expressing photoreceptors exposed to light. Subsequently, it was postulated that the endolysosomal defects of these photoreceptors are exacerbated when exposed to light, due to bulk endocytosis of rhodopsin. To test this theory, the formation of rhodopsin was blocked by raising flies without vitamin A. Inhibiting rhodopsin formation dramatically rescued light-dependent photoreceptor degeneration. Experiments are underway to block the firing of action potentials in amyloid-expressing photoreceptors by co-expressing an inward-rectifying potassium channel. This will differentiate between degeneration caused by rhodopsin endocytosis and that caused by neuronal activity. Additionally, the endolysosomal defect observed in amyloid-expressing photoreceptors is being characterized by means of colocalization studies. To identify the organelles containing rhodopsin accumulations, immunofluorescent staining for endosomes, lysosomes, and autophagosomes will be carried out. Findings thus far indicate that the presence of amyloid-beta does exacerbate endolysosomal dysfunction and neurodegeneration. By characterizing the exact defect in this pathway involved

in rhodopsin accumulation, information gleaned will shed light on the underlying flawed mechanisms in AD pathology. Such insights will inform a more detailed understand of AD development and progression. This will in turn allow medical researchers to identify and or advance novel therapeutics targeting these defects, providing a much needed solution to a tragic disease with few effective therapies available to treat victims.

Introduction

Alzheimer's Disease:

Alzheimer's disease (AD) is a neurodegenerative disease in which structural changes occur in the brain in conjunction with neuronal death. This neurodegeneration typically occurs over the course of an individual's lifetime, the average age of onset of AD symptoms is 65 years of age. Subsequently, AD is known as an aging disease as incidence of diagnosis soars in direct relation to age. For example, approximately 50% of individuals aged 85 and older in the US are diagnosed with AD (Casey et al. 2010). Neurodegeneration associated with AD causes dementia which is the loss of basic social and thinking skills. Other symptoms of AD are memory loss, disorientation, non-memory cognitive deficits, and visuospatial issues. These symptoms typically begin mildly and progress from moderate to severe over the course of several years (National Institute on Aging, 2016).

In 2017, 5.3 million Americans were diagnosed with AD. 200,000 of those individuals were under 65 and had been diagnosed with familial early onset AD in the United States. Familial early onset AD is a subset of this disease caused by genetic mutations. However, the majority of AD cases are late onset type and are not thought to be solely the result of genetic mutation. Worldwide, AD afflicts 44 million people, and its incidence is projected to rise markedly in the future given the increase in the rate of diagnosis in the past twenty years. As AD

progresses, victims require assistance carrying out basic activities, such as dressing oneself. This creates an exorbitant demand for care of AD victims. It is estimated that 15 million Americans provide unpaid care for victims of AD. This brings the medical cost of AD in the US to \$230 billion in 2017 alone due to lost wages associated with providing unpaid care to loved ones, as well as the cost of professional and medical assistance (Alzheimer's Association, 2017). The rising prevalence of AD places a severe financial and medical burden across our nation and the world. Such need demands medical treatments that are effective in prolonging and improving victims' quality of life, cognition, and overall mental well-being.

Current Treatments:

The damage incurred to the brains of AD victims is irreversible and progressive. Neurodegeneration cannot be reversed because currently there is a paucity of knowledge regarding how to regenerate neurons in the human brain. At this time, there are only drugs approved in the United States which act as disease-modifying therapies for treatment of AD. Specifically, these drugs target molecules to increase or decrease the activities of certain neurotransmitters in the brain thought to maintain mental function and temper behavioral issues associated with AD. To date, there are five drugs approved by the Food and Drug Administration (FDA) to be used in the treatment of AD in the US. Four of these drugs are Cholinesterase Inhibitors (CI) which increase the amount of available acetylcholine, a neurotransmitter associated with learning and memory used to transduce signals between the synapses of neurons. CIs are typically used in the mild to moderate stage of AD. Donepezil, brand name Aricept, is the only CI that has been approved by the FDA to be used in the severe stages of AD. The fifth drug available is a NMDA receptor antagonist which acts to reduce the amount of glutamate

signaling in synapses, thought to help slow the progression of neuronal death in moderate to severe stages of AD (Casey et al. 2010).

Although there are drugs which have shown to have some efficacy in the treatment of symptoms associated with AD, effectiveness of these drugs has not proven to be very strong. Patients with mild AD symptoms prescribed CIs show a 1.5 point increase in the Mini Mental State Examination (MMSE) test, an exam used to assess overall cognitive function. However scores on the MMSE drop below baseline six to nine months after starting treatment. Additionally, CIs are reported to have gradual dose escalation, in which a greater dose of drug is required to yield a therapeutic effect, but simultaneously induces a greater magnitude of undesirable side effects such as nausea and diarrhea (National Institute on Aging, 2016). It is estimated that only 50% of subjects show any benefit to treatment with CIs. Furthermore, Donepezil shows no benefit in comparison to placebo in clinical trials attempting to modulate agitation, a symptom common in AD. In sum, drugs available for the treatment of AD are merely palliative in their benefits, which are limited for many patients. This lack of effectiveness, in combination with the cost of these drugs, which is estimated on average to be \$1800 per year, leaves much to be wanted in terms of treatment of AD (Casey et al. 2010). These issues underscore the significance of biomedical research being carried out today aimed at elucidating the causes of AD. Information derived from these studies can identify novel targets for therapeutics as well as potential mechanisms to stop neurodegeneration all together.

Pathology of Alzheimer's Disease

Neuronal dystrophy and death, vascular alterations, and abnormal deposition of neurotoxic proteins are observed in the brains of AD victims (De Strooper and Karren, 2016). These protein aggregates form two different structures, plaques and tangles (Figure 1). Plaques

are composed predominantly of A β 42 protein, a derivative of a generally nontoxic transmembrane protein Amyloid Precursor Protein (APP). A β 42, or amyloid-beta, is an insoluble oligomer that accumulates extracellularly in the central nervous system (CNS) and the cerebral cortex. Neurofibrillary tangles are intracellular structures composed of hyperphosphorylated tau, a protein involved in the stabilization of microtubules (Casey et al. 2010). It is thought that aggregation of these proteins and neuronal death precedes onset of symptoms by several decades (De Strooper and Karren, 2016).

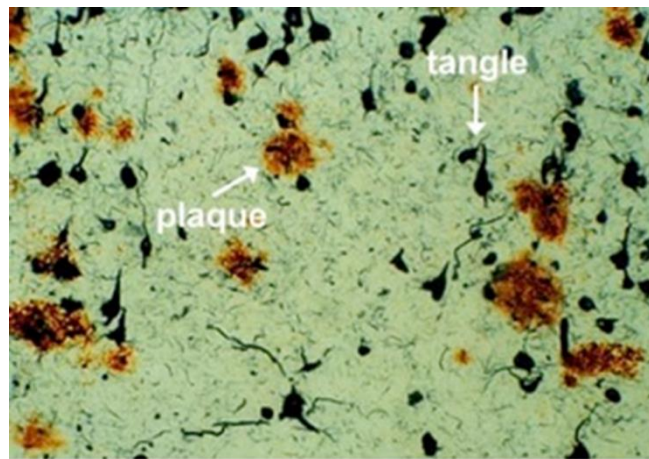


Figure 1. Visualization of plaque and neurofibrillary tangle accumulations in the brain of an AD victim adapted from LaFerla and Dick-Muehlke, UC Irvine Institute for Neurological Impairments and Neurological Disorders.

The relationship between aggregation of A β 42 and tau and neurodegeneration is ill-defined. The predominate theory in this field, the amyloid cascade hypothesis emphasizes amyloid-beta accumulation as the causative force responsible for neurodegeneration. More specifically, this hypothesis postulates that the increased formation of insoluble A β 42 oligomers leads to its extracellular aggregation in the CNS. Such aggregation leads to neuronal dysfunction and death and subsequent formation of neurofibrillary tangles. A strength of this hypothesis is that it brings together the pathology and symptoms of AD, as well as genetic mutations associated with familial AD. However, this theory hypothesizes a linear set of events which have

been found to not necessarily true in human and mouse model studies. Additionally, any potential interaction between amyloid-beta and tau accumulations has been ignored (Karren et al. 2011). Therefore, there is incomplete evidence and information as to the exact relationship between amyloid and tau accumulations and neuronal death in AD. Further work must be carried out to define these relationships to further provide insight on the etiology of this disease.

How toxic proteins are degraded

An important factor to consider when investigating amyloid-beta and tau protein accumulations is the mechanisms cells use to degrade toxic proteins. Cells degrade material by the autophagy-lysosomal system (ALS) or the ubiquitin-proteasome system (UPS) (Ghavami et al. 2014). In the UPS, proteins are targeted for degradation by means of an ubiquitin tag which targets them to the proteasome. These proteins are then cleaved into their amino acid monomers by the proteasome (Figure 2). Autophagy is carried out when a cell engulfs and digests part of its intracellular material. This process is thought to be induced by starvation or stress signals propagating throughout the cell. Upon engulfment of material an autophagosome is formed which fuses with the lysosome to be digested by low pH and hydrolytic enzymes (Nixon et al. 2005). Additionally, in the endocytic pathway extracellular organic material is engulfed in to a vesicle called an endosome. The endosome undergoes various internal changes through the actions of trafficking proteins to form an intermediate and then late endosome. The late endosome fuses with the lysosome which degrades its contents (Figure 3). Both the UPS and ALS degrade intracellular organic material while the endocytic pathway intakes and eliminates extracellular substrates. The UPS is more limited in the substrates, primarily proteins, it can degrade in comparison to the ALS. Furthermore, the ALS sequesters its substrates in membrane-bound vesicles which undergo a maturation process to eventually fuse with the lysosome, a process not

entirely understood. Meanwhile, the UPS covalently modifies proteins thereby targeting them for degradation.

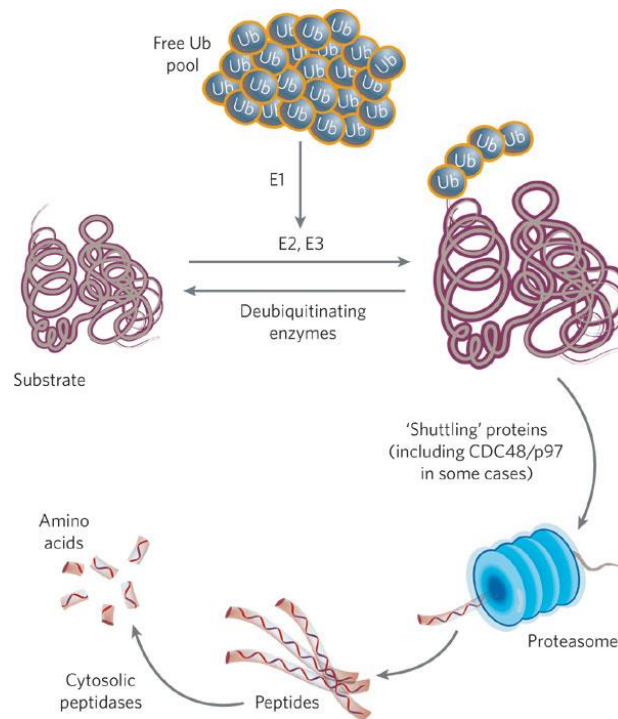


Figure 2. Schematic of series of events carried out in ubiquitin-proteasome system (UPS) adapted from Rubinsztein (2006). Prior to trafficking to the proteasome proteins are covalently modified with ubiquitin. This modification is carried out by ubiquitin-activating, ubiquitin conjugating, and ubiquitin ligase enzymes, termed E1, E2, and E3 respectively in this diagram. Proteins tagged with ubiquitin are trafficked to the proteasome by chaperone proteins CDC48/p97. Upon arriving at the proteasome, proteins are broken into their peptide sequences and then degraded to their amino acid monomers by cytosolic peptidases (Rubinsztein, 2006).

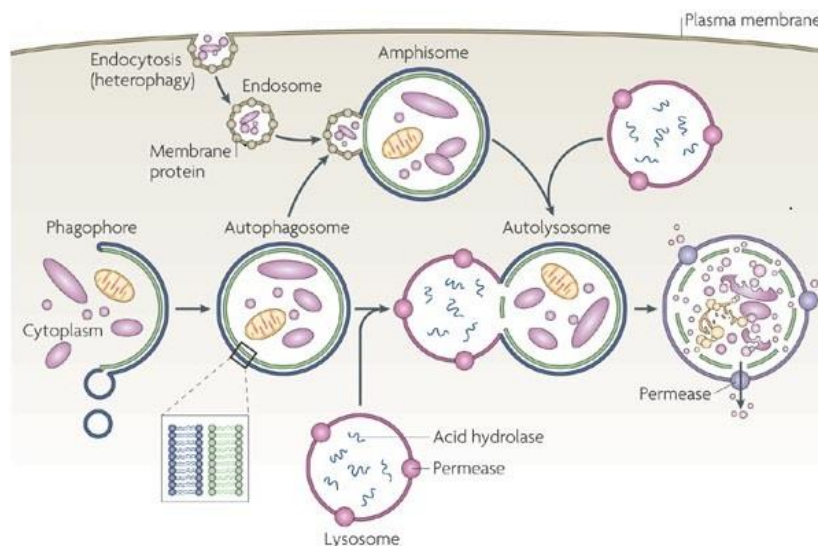


Figure 3. Schematic of the processes carried out in the autophagy-lysosome system (ALS) adapted from Castro-Obregón (2010). Autophagy is carried out initially by engulfment of cellular material to form a phagophore. Intracellular changes occur in the phagophore through a process of maturation, forming an autophagosome. The autophagosome can then fuse with the lysosome to form an autolysosome or with late endosomes to form an amphisome. The endocytic pathway converges into this the autophageal pathway. Early endosomes sequestering extracellular material and undergo a process of maturation. Late endosomes fuse with the lysosome and their contents digested (Castro-Obregón, 2010).

Given that amyloid-beta and tau are accumulating in the brain in AD, either the UPS or ALS, or both of these protein degradative systems are defective. Amyloid-beta protein cannot be degraded by the UPS because it is such an organically stable oligomer. This leaves amyloid-beta to be degraded by the ALS (Ihara et al. 2012). Interestingly, studies have found autophagosomes in brain sections of AD victims, which are not normally found in the CNS of healthy individuals. These studies have also found autophagosomes to be adjacent to amyloid-containing plaques (Figure 4). Furthermore, in Presenilin 1/Amyloid Precursor Protein (PS1/APP) mice, a model of familial AD, both presenilin and amyloid-beta peptides immunocolocalize with autophagosomes (Figure 5). This is thought to be the result of amyloid-beta peptides being generated from the breakdown of APP-containing organelles by autophagy. Researchers have hypothesized that in AD autophagosome maturation is somehow inhibited, increasing the amount pre-lysosomal autophagosomal intermediates in the neurons leading to failure of the ALS and cell death (Nixon, 2007). This highlights the question of whether the autophagosomes are a response to amyloid accumulation, or are somehow impeded thereby resulting in the inhibition of plaque degradation. This evidence supports the theory that autophagy is involved in AD pathogenesis, but to what capacity it remains unknown.

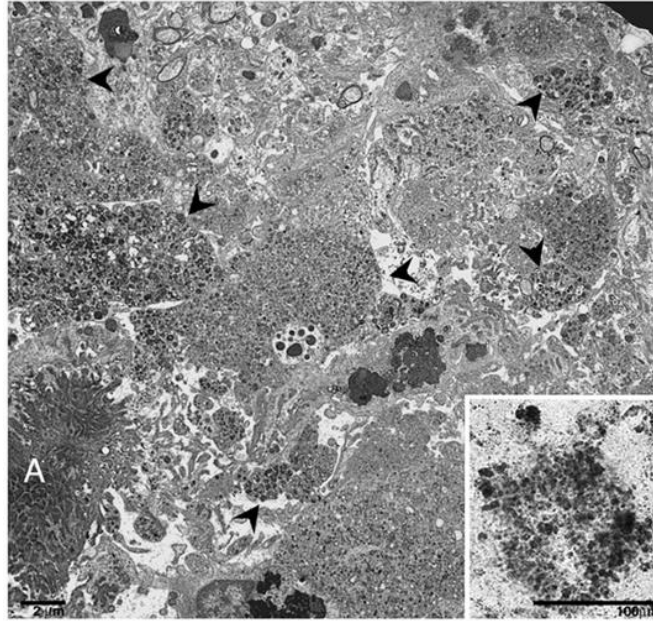


Figure 4. Autophagosomes are visualized to be in close vicinity to amyloid plaques and sites of neuronal dystrophy in AD victims' brains adapted from Nixon et al. (2005). Autophagosomes were visualized via electron microscopy using Ig-gold in the cross section of the brain. The accumulation of these vesicular bodies near plaque formations and neuronal loss are marked with arrowheads (Nixon et al. 2005).

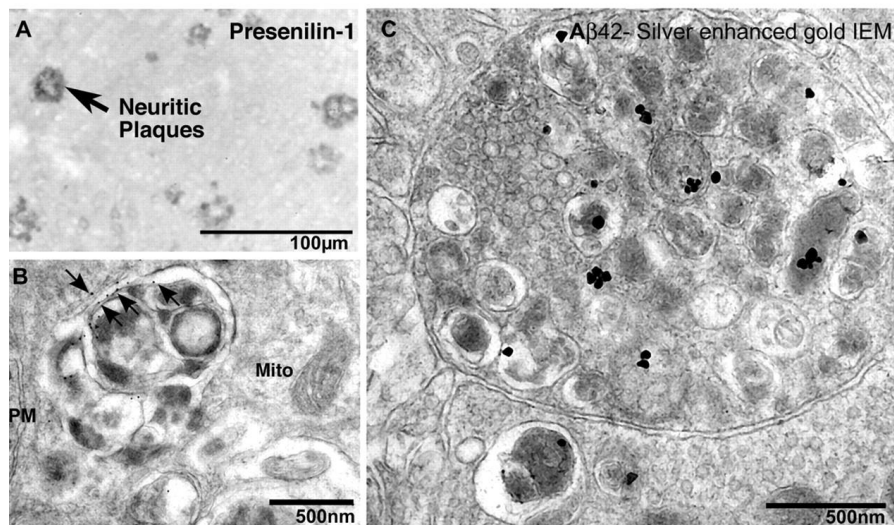


Figure 5. In PS1/APP mouse brains amyloid-beta peptide and presenilin 1 have been found to colocalize with autophagosomes adapted from Nixon (2007). A. Antibodies conjugated to gold particles localize to PS1 within amyloid-containing neuritic plaques. B. Immuno-gold staining of PS1 demonstrates that this protein is present on the membrane of autophagosomes. C. Silver-enhanced Ig-gold staining of Aβ42 (shown in dark black) localizes within autophagosomes, the distinctive vesicles outlined, in dystrophic neurons (Nixon, 2007).

Experimental Questions

This study focused on the following questions probing the role of amyloid-plaque formation in AD: Given the protein degradative machinery in our cells, how is A β 42 aggregating? Furthermore, how does the accumulation of A β 42 lead to neurodegeneration? Specifically, this experimentation explored whether the ALS is defective in the presence of A β 42 given recent evidence of the role of autophagy in this disease. Additionally, an aim of this study was to identify the exact defect in this system that leads to plaque formation. In aiming to understand these questions the link between neurotoxic amyloid protein aggregation and neuronal death can be further defined. Information generated from these studies can be used to determine novel targets or strategies to treat AD.

Experimental Design

To carry out experimentation, a *Drosophila melanogaster* (fruit fly) animal model was used to model A β 42 accumulation in neurons. This model was used given that *Drosophila* is an established model system and its well-defined genetics. Additionally, gene manipulation can be carried out simply and effectively using transgenes. Moreover, fruit flies are easy to care for and have a fast generation time of approximately nine days. The neuron of the fruit fly also possesses similarity to that of the human neuron (Bellen et al. 2010). Consequently, the neurons of the fly eye, or photoreceptors, expressing A β 42 were used as a model of a human neuron to understand the how this protein affects the ALS and overall neuronal health. By studying this aspect of AD and defining the relationship between amyloid-beta protein and the ALS, insights can be gained regarding AD pathogenesis. A weakness of this model system is that it is an artificial model of AD as this disease is much more complex in terms of proteins and cell populations involved in neuronal dystrophy and death.

Another reason the fly eye was chosen is due to the established lysosomal stress of light in *Drosophila melanogaster*. Light induces stress in the ALS because it is thought that this light exposure induces the increased endocytosis of rhodopsin, a G-Protein Coupled Receptor (GPCR) protein associated with the structure of the retina. It has been proven that *Drosophila* neurons with impaired lysosomal function degenerate when they are overwhelmed by rhodopsin (Chincore et al. 2009). In this study, it was hypothesized that the presence of amyloid-beta protein in *Drosophila* sensitizes photoreceptors to endolysosomal defects when exposed to light due to bulk endocytosis of rhodopsin. From this hypothesis it was predicted that exposing amyloid-expressing flies to light would exhibit more neuronal degeneration than those flies reared in the dark. Further studies focused on the role of rhodopsin in A β 42-induced degeneration (A full description of methods can be found in Materials and Methods section).

Results

Light makes amyloid-beta more toxic to photoreceptors

To assess whether A β 42 makes photoreceptors more sensitive to the toxic effects of light, GMR-Gal4 \rightarrow A β 42 expressing flies were raised for three weeks in either a light or dark condition. Microdissection, immunofluorescent staining and imaging were carried out. The remaining rhabdomeres per ommatidium, each discrete facet of the fly eye, were counted to score neurodegeneration. Rhabdomeres, which are seven light sensing rhodopsin-containing structures, were visualized using phalloidin, a fluorescent dye which binds to cortical actin. To determine whether the presence of A β 42 in the flies manifested some toxicity and was not the result of just light exposure alone, both the light and dark conditions were compared to a negative control, a wildtype fly which did not express transgenes. Staining of the eye showed that the wildtype fly displayed no neurodegeneration after aged in the light for three weeks

(Figure 6, A). Comparing this control to the amyloid-expressing fly in the dark condition, some neurodegeneration is seen with an average of 6.22 ± 0.16 rhabdomeres remaining for (Figure 6, B). This difference demonstrates that A β 42 induces some kind of toxic effect on the flies, indicating that amyloid-beta expression was successfully modeled in the fly. For amyloid-expressing flies raised in the light condition, increased neurodegeneration was observed with only 5.36 ± 0.14 rhabdomeres remaining (Figure 6, C).

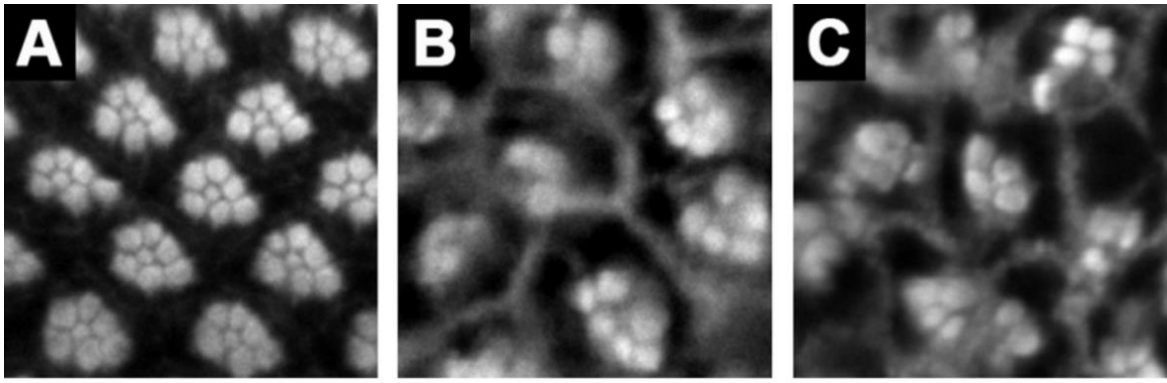


Figure 6. Amyloid-expressing flies raised in the light show more degeneration than those raised in the dark. A. Eye from a wild type fly which acted as a negative control. The ommatidia are organized and each facet contains seven discrete units, rhabdomeres, indicating little if any degeneration has taken place. B. Eye from an amyloid-expressing fly raised in the dark. The structure of the eye is less organized and there are approximately six rhabdomeres per ommatidia. C. Eye from an amyloid-expressing fly raised in the light. The structure of the eye is fairly disorganized and there are visually four to five rhabdomeres per ommatidia.

Analysis of the neurodegeneration observed in these light and dark conditions in the amyloid expressing flies found that the difference between these two groups to be statistically significant, $p = 0.0009$ (Figure 7). This finding indicates that the presence of amyloid in the light does induce a significant increase in photoreceptor degeneration.

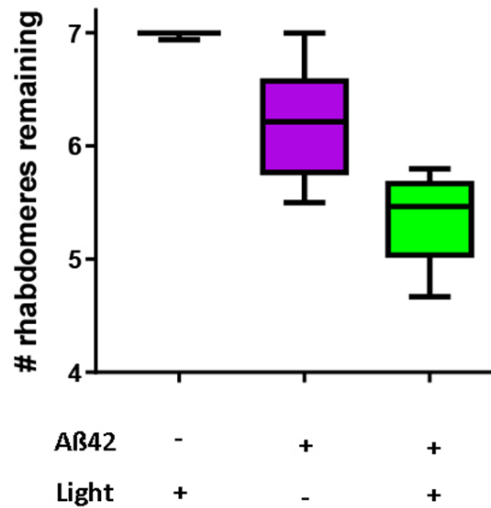


Figure 7. Scoring of neurodegeneration of amyloid-expressing flies between light and dark conditions and with and without amyloid-beta expression. Neurodegeneration was scored by number of rhabdomeres per ommatidia left. The box and whiskers represents the interquartile range of the data with lines representing the minimum, average, and maximum values of the data set. There is no neurodegeneration in the negative control. There are on average 6.22 ± 0.16 ($n = 10$ eyes) rhabdomeres per ommatidium remaining for the amyloid-expressing dark reared flies. There are 5.36 ± 0.14 ($n = 8$) rhabdomeres remaining in the amyloid-expressing flies reared in the light. A Two-Tailed Student T-test found the difference between the light and dark conditions was found to be $p = 0.0009$.

Rhodopsin exacerbates amyloid-beta toxicity in photoreceptors

In an effort to understand the contributing sources to the light-dependent degeneration observed, the question was asked whether rhodopsin endocytosis was solely responsible for this cell death. To test this question, rhodopsin formation in the flies was inhibited by raising them on a vitamin A deficient diet. Vitamin A is a required compound needed to form retinol, an opsin protein that is a part of the rhodopsin complex. Therefore, without vitamin A rhodopsin would not form in the flies. Amyloid-expressing flies were raised on a vitamin A deficient diet and the photoreceptor degeneration compared to amyloid-expressing flies raised on normal food for the light condition (Figure 8, B, C). Qualitatively speaking, the structure of the eye is more organized and more rhabdomeres were observed in those flies raised on this medium. Additionally, to confirm that rhodopsin was not formed in these flies, eyes were stained

immunofluorescently for this protein. Little if any rhodopsin stain was observed, indicating that this protein was in fact not formed using this method (data not shown).

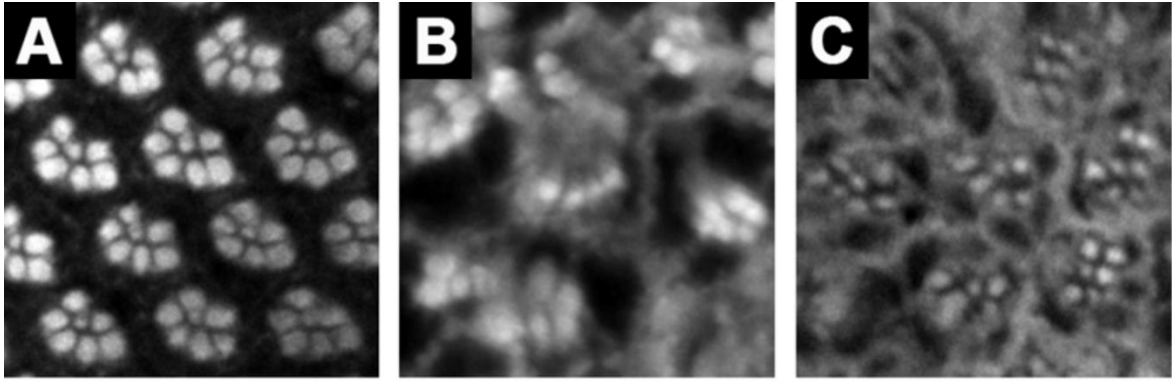


Figure 8. Amyloid-expressing flies raised in the light in the absence of vitamin A exhibit rescue of light-dependent degeneration. A. Eye from a wild type fly reared in the light which served as a negative control. The structure of the ommatidia is organized and each facet contains seven discrete units indicating no degeneration has occurred. B. Eye from an amyloid-expressing fly in the light raised on normal food exhibiting significant degeneration and disorganization of the eye overall. C. Eye from an amyloid-expressing fly raised in the light on a vitamin A deficient diet. Although small, there are six to seven rhabdomeres present showing little degeneration has taken place.

Statistical analysis indicates that inhibition of rhodopsin formation in photoreceptors significantly rescues the light-dependent degeneration found in the amyloid-toxicity experiment ($p < 0.0001$). On average, amyloid-expressing flies raised on vitamin A deficient media in the light had 6.779 ± 0.058 rhabdomeres remaining (Figure 9). Such a drastic rescue of this light-dependent degenerative phenotype demonstrates the significance of rhodopsin as a mediator in neuronal loss in this model.

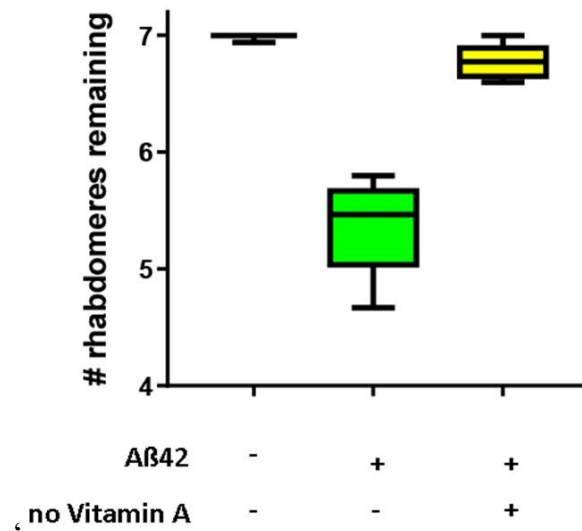


Figure 9. Comparison of neurodegeneration between amyloid-toxicity experiment and amyloid-expressing flies reared without rhodopsin. Neurodegeneration was scored by counting the rhabdomeres left per ommatidium. The box and whisker plots for each condition show the interquartile range for each condition containing the minimum, average, and maximum values. There is no neurodegeneration in the negative control, wild type flies raised in the light. There is significant degeneration in the amyloid-expressing flies reared in the light with an average of 5.36 ± 0.14 ($n = 8$ eyes) rhabdomeres remaining. The amyloid-expressing flies raising in the light on vitamin A deficient food showed significant rescue in neurodegeneration with an average of 6.779 ± 0.058 ($n = 6$) rhabdomeres remaining. The difference between the normal food light-reared flies and the vitamin A deficient flies was found to be statistically significant, $p < 0.0001$, using a Two-Tailed Student's T test.

Rhodopsin accumulation in the endolysosomal pathway in light-reared flies

In the amyloid-toxicity experiment, eyes from both light and dark conditions were immunofluorescently stained with for rhodopsin. In the light condition, amyloid-expressing flies exhibited rhodopsin puncta within and around ommatidia. This intensity of staining was not observed in the dark condition, demonstrating that in the light rhodopsin is accumulating somewhere in the endolysosomal system. This reveals a defect which is impairing degradation of this protein. This observation further supports the hypothesis that the ALS and endolysosomal system in amyloid-expressing flies are defective (Figure 10).

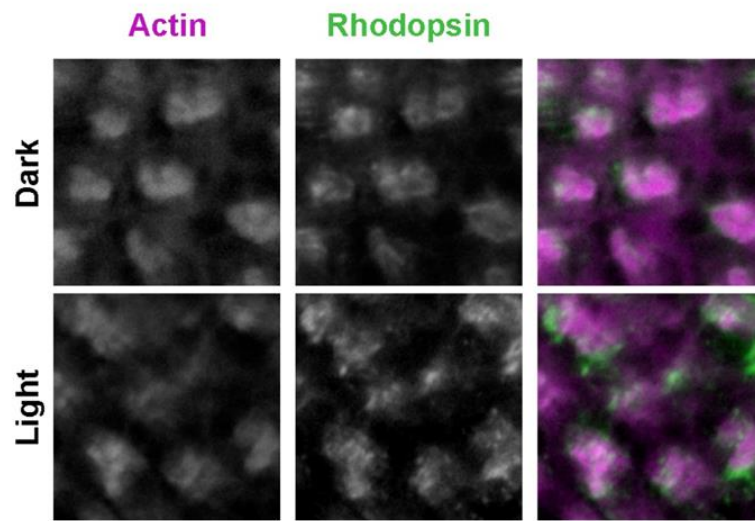


Figure 10. Rhodopsin puncta are observed in light-reared amyloid-expressing flies. Amyloid-expressing eyes were stained for actin and rhodopsin. Actin was stained for using phalloidin and is shown in magenta. Rhodopsin is shown in green. Actin staining is shown in the far left panel for each of the conditions, the middle rhodopsin. An overlay of these stains is shown on the far right. Comparing the light and dark conditions, there are accumulations of rhodopsin in the light, and little if any in the dark condition.

Future Directions

Two hypotheses of light-dependent neurodegeneration

The findings of this study point to the significant involvement of rhodopsin in the formation of a light-dependent neurodegenerative phenotype in this model. At this time, the exact mechanism by which rhodopsin mediates neurodegeneration is unknown. Two alternate hypotheses have been postulated to describe the events leading to this neurodegeneration (Figure 11). The first is the Rhodopsin endocytosis hypothesis in which light induces rhodopsin to be endocytosed and targeted for degradation by the endolysosomal system. However, over time rhodopsin overwhelms this system with the added stress of A β 42 expression impairing this pathway, leading to lysosomal dysfunction, failure, and neuronal death. Alternatively, the potential role of neuronal firing must be considered in mediating cell death in this model. Consequently, the second hypothesis is a Neuronal firing hypothesis in which light induces rhodopsin to undergo a conformational change and be “excited,” which in turn causes an action

potential to fire by the neuron to transmit a chemical message. Continual firing over time causes the neuron to age, and it eventually burns out and dies.

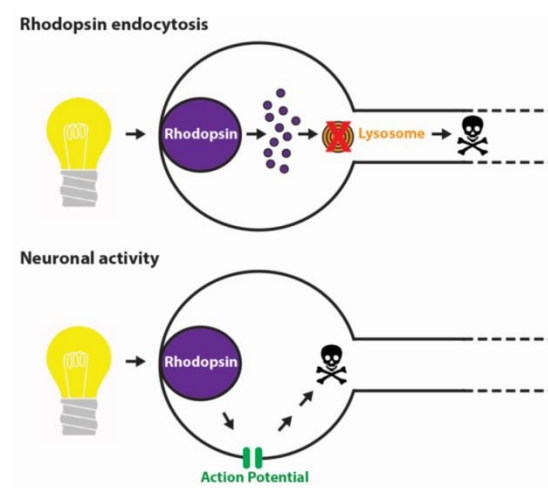


Figure 11. Two alternate hypotheses proposed for the mechanism of light-dependent neurodegeneration. The Rhodopsin endocytosis hypothesis postulates that light induces rhodopsin to be endocytosed from the cellular membrane into the intracellular environment of the cell. The presence of A β 42 impairs the ALS causing lysosomal dysfunction as it is overwhelmed by bulk rhodopsin endocytosis. Lysosomal failure leads to death of the neuron. In the Neuronal activity hypothesis light is proposed to cause rhodopsin to be excited and targeted for the endocytic pathway at the cellular membrane. The excitation of rhodopsin leads to the firing of an action potential by the neuron. Over time this firing of the neuron leads to neuronal dysfunction and death.

At this time, these two hypotheses cannot be differentiated. An experiment to characterize the potential involvement of neuronal firing has been planned. In this experiment Kir 2.1-expressing flies will be bred with GMR-Gal4 \rightarrow A β 42 expressing flies. Kir2.1 is an inward rectifying potassium channel in neurons. In Kir2.1-expressing flies normal polarization of the neuron is inhibited, thereby preventing neurons from firing (Hodge, 2009). Subsequently, breeding GMR-Gal4 \rightarrow Kir2.1 to GMR-Gal4 \rightarrow A β 42 individuals, the progeny from this cross will express amyloid-beta in their eyes and their photoreceptors will not fire. These flies will be reared in the two light conditions and aged for three weeks. Eyes will be dissected, stained, and imaged as was done in the experiments carried out in this study. By inhibiting neuronal firing it can be determined to what capacity neuronal activity is contributing to the light-dependent

neurodegenerative phenotype. Some predictions for this experiment are that if similar light-dependent degeneration is found for the light condition as found in the amyloid toxicity experiment, then neuronal firing is most likely not involved in this cell death. Such a finding would further support the Rhodopsin endocytosis hypothesis. Instead, if rescue of neurodegeneration is exhibited in the light condition, then this result would suggest that neuronal activity is involved, thereby supporting the Neuronal firing hypothesis.

Localization of rhodopsin accumulations in the endolysosomal system

As discussed in the results, rhodopsin was observed to be accumulating at increased levels in the light reared amyloid-expressing flies compared to the dark-reared individuals. Such a finding suggests a failure of the endolysosomal system. In order to identify the exact defect in this system studies have been proposed to replicate the amyloid-toxicity experiment and stain eyes with immunofluorescently for rhodopsin and markers of different compartments involved in the endolysosomal system. These compartments include fluorescent antibodies targeting the late endosome, autophagosome, and lysosome. The purpose of this staining will be to identify which compartment, or compartments, rhodopsin colocalizes with. Therefore, the location of rhodopsin accumulations can be elucidated and a detailed mechanism of defect can be proposed.

Verification by electrophysiological methods

In order to verify the neurodegeneration quantified in these studies, verification by electrophysiological methods must be carried out. An electroretinogram (ERG) is a test in which electrodes monitor and record the electromagnetic field potential of the fly eye. Typically ERGs are measured in response to light and quantitation of visual transduction can be assessed by analyzing the differences in electromagnetic field potential through time (Vilinsky and Johnson, 2012). Therefore, the amount of photoreceptor firing can be quantified in wildtype flies in

response to light and then be compared to amyloid-expressing flies both light and dark-reared. The recorded differences between the firing of these eyes will enable quantitation of cell death. This method of scoring of neurodegeneration will be performed for both the amyloid toxicity and the vitamin A deficient flies to verify conclusions drawn from the microscopy findings. Additionally, when carrying out the Neuronal firing hypothesis experiment, lack of neuronal firing in the Kir2.1-expressing can be verified.

Conclusion

Significance of findings for the fly eye model

In terms of analyzing the results in the context of the fly eye model used, rhodopsin-mediated light-dependent degeneration appears to be specific to photoreceptors. There are approximately 100 peer reviewed articles published to date that use the fly eye model in studying neurodegenerative disease. Subsequently, the findings from this study highlights the conclusion that the scoring of neurodegeneration in these studies could be skewed by light as it could have had confounding effects on the results.

Reflecting more on the fly eye as model in relation to the study aims, the aspect of artificiality of this model must be accounted for. First, although a fly neuron is similar to that of a human neuron, the two are not equal in all aspects. Secondly, the amyloid-expressing fly does not account for the complexity of Alzheimer's disease. Primarily, it must be emphasized that other proteins, such as hyper-phosphorylated tau, accumulate in AD. Additionally, there are many cell populations, such as glial cells, involved in the physiological changes occurring in the brain which are not addressed by this model. Rather, this model allows us to analyze the specific relationship between amyloid-beta and neuronal health. Additionally, a detail to note is that

rhodopsin acts as a stressor on the ALS in this model, rhodopsin is not relevant to AD pathogenesis in humans.

Significance of findings to AD pathogenesis

In the context of AD pathogenesis, the results of this study hold important implications for the treatment of AD patients and at-risk individuals. The findings do support the endolysosomal defect hypothesis initially postulated to the extent that amyloid-beta does appear to exacerbate endolysosomal defects present in the ALS when subject to stress. However, it is unclear at this time whether rhodopsin mediates neuronal death by ALS dysfunction or by mode of neuronal firing. The proposal of the Neuronal firing alternative hypothesis holds serious implications should neuronal activity is found to be involved neurodegeneration in this model. Specifically, the popular belief in the field of neuroscience is that a neuron does not die by means of use, but rather disuse. The Neuronal firing hypothesis is a contrary burn-out theory model. Typically individuals at risk for developing AD are told to “exercise” their brain such as doing puzzles, reading, and other activities that require cognitive exertion. If neuronal firing does prove to be involved in neurodegeneration, how the medical field treats at-risk individuals for AD will need to change.

The proposed experiments to localize where rhodopsin is accumulating in the endolysosomal system will shed light in the impairment in the ALS in the model. By identifying this defect, this information will allow us to better understand neurotoxic protein accumulation in AD. By defining this exact defect in the ALS, the findings from these experiments will enable superior therapeutics to be identified. For example, Rapamycin is a drug used to coat coronary stents, as well as has immunosuppressive effects in humans (American Chemical Society, 2016). Rapamycin has also been found to induce autophagy in cells (Cai, 2013). If conclusions from

this study reveal an inability for autophagosomes to mature and fuse with the lysosome, then treatment of AD with Rapamycin holds promise because this drug targets and enhances this pathway. This study and future experiments proposed have significant consequences for the identification of therapeutics which will aim to treat the underlying pathology of AD, rather than those that are merely symptom-modulating. This work will provide information to advance more meaningful options to the millions of victims of AD in the United States and around the world.

Materials and Methods

A transgenic A β 42 fruit fly was the experimental model used to carry out this study. This fly was made by breeding GMR-Gal4 expressing virgin female flies to UAS-A β 42 expressing males. GMR-Gal4 drives expression of transgenes specifically in the fly eye. The UAS-Gal4 driver system is widely used in the scientific community to induce local expression of transgenes in *Drosophila*. The Gal4 gene encodes for a yeast transcriptional activator protein and directs transcription of genes downstream of the Upstream Activating Sequence (UAS), in this case the gene that codes for A β 42 protein (Li et al. 2012). This cross produced a fly with expression of GMR-Gal4 \rightarrow A β 42 in its eye. Localized expression of A β 42 was chosen for this study because global expression of amyloid-beta induces toxic effects that significantly shorten the lifespan of these flies (Prüßing, Voigt, & Schulz, 2013). GMR-Gal4 and UAS-A β 42 stock flies were purchased from Bloomington Drosophila Stock Center at Indiana University.

Initially, an experiment was carried out to assess amyloid toxicity in the fly eye. GMR-Gal4 \rightarrow A β 42 progeny were collected at one day old and raised in two different conditions. One condition was the “light condition” in which the flies were exposed to 12 hours of light and 12 hours of dark per day. The other condition was the “dark condition” in which the flies were aged in 24 hours of dark per day. The flies were aged for three weeks at 25° Celsius. The vials these

flies were aged in were changed once a week to resupply food (Bloomington Standard Medium) and prevent these flies from being mixed with their progeny, whose genotype was not known. In addition to assaying these flies, a negative control, a fly that expressed no transgenes was assessed as well. This control aimed to answer the question whether significant degeneration occurs in the light condition of healthy flies, and therefore if our method of scoring degeneration was confounded by light alone.

After three weeks flies from both the light and dark conditions expressing amyloid-beta and the wildtype controls were put under using carbon dioxide gas, their eyes were then microdissected in 3.7% paraformaldehyde fixative solution. After fixing eyes while shaking for 30 minutes, eyes were washed with 1X PBS, further dissected to get rid of excess cuticle and stored in 1X PBS-Triton. The eyes were immunofluorescently stained with 1:500 dilution of TRIC-phalloidin, and 1:50 dilution of Rhodopsin 4C5 mouse antibody from the Developmental Studies Hybridoma Bank. Secondary goat anti-mouse 488 and goat anti-rabbit 647 antibodies from Abcam were used following primary staining in 1:500 dilutions. After completion of staining, eyes were mounted on slides using VectaShield. The eyes were imaged using an Olympus A-1 R confocal microscope in which 10 μ m Z-planes were taken to obtain the most comprehensive view of the cells present.

After imaging, the number of rhabdomeres, seven visible light sensing structures, per ommatidium (facet of the eye) were counted using ImageJ software by three individuals to ensure consistency and accuracy of results. The average number of rhabdomeres per ommatidium left for each eye was calculated for both conditions (light and dark for both amyloid-expressing and wildtype flies). This data was used to perform a Two-Tailed Student T-test to assess the differences between the conditions.

Following the amyloid toxicity experiment, a follow up experiment was carried out to assess whether rhodopsin was responsible for the light-dependent degeneration observed. Rhodopsin formation was prevented by raising GMR-Gal4→Aβ42 expressing flies on vitamin A deficient food. Vitamin A deficient food was made according to Nichols and Pak, 1985 (Nichols and Pak, 1985). Then the UAS- Aβ42 x GMR-Gal4 cross was carried out in these food vials for both light and dark conditions. GMR-Gal4→Aβ42 expressing progeny were isolated at one day old and then aged in their in light and dark conditions for three weeks at 25° Celsius. Following aging, microdissection of the eyes was carried out, followed by staining and imaging as previously described. This data was statistically analyzed using a Two-Tailed Student T-test to assess the differences between the conditions. The results of this experiment were compared to the amyloid toxicity experiment using a T-test.

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Reference List

- American Chemical Society. (2016). Rapamycin. Molecule of the Week.
- Alzheimer's Association. (2017). Alzheimer's Disease Facts and Figures. Alzheimer's Dementia, 13: 325-373.
- Bellen, H. J., Tong, C., & Tsuda, H. (2010). 100 years of Drosophila research and its impact on vertebrate neuroscience: a history lesson for the future. *Nature Reviews Neuroscience*, 11(7), 514-522.
- Cai, Z., & Yan, L. J. (2013). Rapamycin, autophagy, and Alzheimer's disease. *Journal of biochemical and pharmacological research*, 1(2), 84.

- Casey, D. A., Antimisiaris, D., & O'Brien, J. (2010). Drugs for Alzheimer's disease: are they effective?. *Pharmacy and Therapeutics*, 35(4), 208.
- Castro-Obregón, S. (2010). The discovery of lysosomes and autophagy. *Nature Education*, 3(9), 49.
- Chinchore, Y., Mitra, A., & Dolph, P. J. (2009). Accumulation of rhodopsin in late endosomes triggers photoreceptor cell degeneration. *PLoS Genetics*, 5(2).
<https://doi.org/10.1371/journal.pgen.1000377>
- De Strooper, B., & Karran, E. (2016). The cellular phase of Alzheimer's disease. *Cell*, 164(4), 603-615.
- Ghavami, S., Shojaei, S., Yeganeh, B., Ande, S. R., Jangamreddy, J. R., Mehrpour, M., ... Łos, M. J. (2014). Autophagy and apoptosis dysfunction in neurodegenerative disorders. *Progress in Neurobiology*, 112, 24–49. <https://doi.org/10.1016/j.pneurobio.2013.10.004>
- Hodge, J. (2009). Ion channels to inactivate neurons in *Drosophila*. *Front. Mol. Neurosci*, 2(10.3389).
- Ihara, Y., Morishima-Kawashima, M., & Nixon, R. (2012). The Ubiquitin–Proteasome System and the Autophagic–Lysosomal System in Alzheimer Disease. *Cold Spring Harbor Perspectives in Medicine*, 2(8), a006361. <http://doi.org/10.1101/cshperspect.a006361>
- Karran, E., Mercken, M., & De Strooper, B. (2011). The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. *Nature Reviews. Drug Discovery*, 10(9), 698–712. <https://doi.org/10.1038/nrd3505>
- Li, W. Z., Li, S. L., Zheng, H. Y., Zhang, S. P., & Xue, L. (2012). A broad expression profile of the GMR-GAL4 driver in *Drosophila melanogaster*. *Genet Mol Res*, 11(3), 1997-2002.
- National Institute on Aging. (2016). Alzheimer's Disease: Fact Sheet. Alzheimer's Disease Education and Referral Center. NIH Publication No. 16-AG-6423
- Nichols and Pak. (1985) A simple medium for vitamin A deprivation of *Drosophila Melanogaster*. *Drosophila Information Service*. 61, 195-196.
- Nixon, R. A., Wegiel, J., Kumar, A., Yu, W. H., Peterhoff, C., Cataldo, A., & Cuervo, A. M. (2005). Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study. *Journal of Neuropathology & Experimental Neurology*, 64(2), 113-122.
- Nixon, R. A. (2007). Autophagy, amyloidogenesis and Alzheimer disease. *Journal of cell science*, 120(23), 4081-4091.
- Prüßing, K., Voigt, A., & Schulz, J. B. (2013). *Drosophila melanogaster* as a model organism for Alzheimer's disease. *Molecular Neurodegeneration*, 8(35).
- Rubinsztein, D. C. (2006). The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature*, 443(7113), 780-786.

Vilinsky, I., & Johnson, K. G. (2012). Electroretinograms in *Drosophila*: a robust and genetically accessible electrophysiological system for the undergraduate laboratory. *J Undergrad Neurosci Educ*, 11, A149-A157.